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14. ABSTRACT

Conditioned medium (CM)from adipocyte stromal cells (ASC) of WAT of both Glipr1+/+ and Glipr1ï /ï adult males after they were either surgically castrated or shamed (Glipr1+/+ and Glipr1ï /ï +/- surgical castration, total 4 subtypes) were collected and analyzed for presence of secreted growth promoting factors. Effects of CM of ASCs from each 4 subtypes were evaluated using normal human umbilical vein endothelial cells (HUVEC) and mouse prostate cancer cells (RM-9). CM from castrated Glipr1ï /ï ASCs promoted the highest cell proliferation and tubule formation of HUVEC cells, and cell proliferation and invasion of mouse (RM-9) prostate cancer cells compared with ASCs-CM from both shamed Glipr1+/+ and Glipr1ï /ï mice and castrated Glipr1+/+ male mice. Microarray assay results showed higher level of proliferin secreted in the CM-ASCs from castrated Glipr1+/+ and Glipr1ï /ï mice compared to shamed Glipr1+/+ and Glipr1ï /ï male mice and higher level in Glipr1ï /ï compared to Glipr1+/+ CM-ASC. These stimulating effects of cell proliferation, tubule formation, and invasion were reduced when CM-ASC from castrated Glipr1ï /ï ASCs was pretreated with prolierin antibody before the addition to the cells listed above. Thus, we surmise secreted proliferin from WAT promotes cell proliferation and tubule formation of HUVEC and mouse prostate cancer cell invasion.

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Prostate cancer

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PROGRESS REPORT (May 2012 – April 2013)

INTRODUCTION

Prostate cancer (PCa) cells are initially sensitive to hormonal manipulation, and androgen-deprivation therapy (ADT) generally reverses androgen receptor (AR)–dependent growth and proliferation. ADT is one of the main treatment modalities in the clinical management of PCa, but ADT is only palliative, and PCa eventually progresses to an androgen-insensitive stage, i.e., castrate-resistant PCa (CRPC), after a median of 12–20 months. Progression to CRPC is a dynamic process that is incompletely understood as yet. Potential mechanisms contributing to the development of CRPC include selective growth of a preexisting hormone-insensitive population of cancer cells as a result of suppression by androgen ablation of the androgen-dependent cell population; activation of oncogenes; inactivation of tumor suppression genes; and interaction between cancer cells and tumor-associated stroma and tumor-associated macrophages. The object of this research project is to investigate the effect of castration on epididymal white adipose tissue (WAT), ventral prostate (VP) tissue, and adipose stromal cells (ASCs) from male *Glipr1*$^{+/+}$ (WT) and *Glipr1*$^{-/-}$(KO) mice. We are testing our hypothesis that the biologic activity of WAT is affected by castration and that although the acute effects of castration (e.g., GLIPR1 induction) may suppress cancer-promoting adipokines, long-term ADT results in monocyte infiltration and the generation of WAT-associated macrophages (WAMs). WAMs, in turn, produce cytokines and promote the growth and survival of growth factor–expressing ASCs, which enter the systemic circulation and promote PCa progression. An important note is that the prostate, an androgen target organ, is significantly affected by castration and also produces cytokines and cytokine receptors that may, in concert with WAT-derived cytokines, contribute to the progression of already established local tumors. We also hypothesize that Glipr1/GLIPR1 protein regulates castration-induced WAMs and ASCs. Our overarching hypothesis is that castration induces alterations in WAT that promote the development of CRPC. During the second funding period, we have completed the studies proposed in Specific Aim 2.

Key Personnel

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BODY

STATEMENT OF WORK

Aim 1: Identify castration-affected and/or Glipr1-regulated genes in ventral prostate (VP) tissue, epididymal white adipose tissue (WAT), and adipocyte stromal cells (ASCs) using in vivo models.

1. Generate a sufficient number of *Glipr1* wild-type (WT) and *Glipr1* knockout (KO) 12-week-old male mice (1–6 months).
2. Perform the surgical castration experiment using the *Glipr1* WT and KO male mice, and collect VP, WAT, and ASCs on days 3, 14, and 35 after castration (6–9 months).
3. Isolate RNA and perform microarray analyses to characterize genes affected by castration in VP, WAT, and ASCs in *Glipr1* WT and KO male mice (9–12 months).

Aim 2: Study the interactions between ASCs isolated from *Glipr1* WT (ASCs-WT) and KO (ASCs-KO) male mice and human prostate cancer cell lines in vitro and in vivo.

1. Isolate, expand, and prepare a stock of frozen ASCs from *Glipr1* WT and KO male mice (6–12 months).
2. Determine the effect of ASCs on the cell growth rate of human prostate cancer cell lines in vitro (12-18 moths)
3. Determine the effect of ASCs conditioned media on the cell growth rate of human prostate cancer cell lines in vitro (12-18 moths).
4. Analyze and compare cytokine and growth factor profiles in conditioned media produced by ASCs (18-24 moths)

Aim 3: To test the response to surgical castration and systemic GLIPR1-ΔTM in vivo using VCaP xenograft model:

1. Generate orthotopic VCaP tumors in athymic nude male mice and determine the effect of surgical castration on the tumor growth and ASCs infiltration profiles at acute, intermediate and chronic time points.
2. Test the effects of systemic GLIPR1-ΔTM on orthotopic VCaP tumor growth and ASCs infiltration profiles ± surgical castration at acute (3d), intermediate (14d) and chronic time points (35d).

RESEARCH

Materials and Methods

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was isolated from each ASC subtypes as described in previous report, and reverse-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Appropriate forward and reverse primers to detect transcripts of interest were used in the qRT-PCR for cDNA amplification. qRT-PCR was performed using the following primers (Integrated DNA technologies, Coralville, IO); Glipr1;5'-TTTGACCGAAGCTGGTTGTGA-3'(Forward) and 5'-CATCCTTGCTGTGATAGT CTGG-3' (Reverse): PLF (Prolactin family2, subfamily C, member 2);5'-AGCCCCATGAGATG CAATACT-3' (Forward) and 5'-CACTCACTAGATCGTCCAGAGG-3' (Reverse): GAPDH (glyceraldehyde -3-phosphate dehydrogenase);5'-TGTAGACCATGTAGTTGAGGT CA-3' (Forward) and 5'-AGGTCGGTGAACGGATTTG-3' (Reverse); with StepOne Real time PCR system (Applied Biosystems). The quantity of each target was normalized against the quantity of GAPDH.

Western blotting

ASCs were prepared from each subtype and protein concentration was determined. The protein in the conditioned medium was precipitated by application of trichloroacetic acid and then the precipitate was washed with acetone and dissolved in 30 μL of sample buffer. Conventional western blotting protocol was used to detect protein levels of PLF (from Santa Cruz, CA) and Glipr-1 (Ren, Li et al. 2002). α-tubulin (Sigma-Aldrich, St. Louis, MO) was used as a loading control. Computer assisted quantitative analysis was done with Nikon's NIS-Elements AR 3.0 imaging and quantification software.

Cell viability assay

Cells were plated in 96-well plate with each appropriate growth medium with 1% FBS at a density of 3×10^3 for RM-9 or $5x10^3$ for HUVEC. Next day, the growth medium from each cell line was aspirated completely, and then ASC-CM was added to each well. After 48h incubation, the number of living cells was measured using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous One-solution Cell Proliferation assay)(Promega, Madison, WI) according to the manufacturer's instruction. Absorbance at 490 nm was measured using a multiwell plate reader (Synergy2, BioTech Instruments, Inc., Winooski, VT).

For PLF neutralization assays, ASC-CM of each subtype was incubated with anti-PLF antibody (50μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA), normal IgG antibody (50μg/ml) (Santa Cruz) or phosphate buffered saline (PBS) and was mixed gently overnight at 4 ºC. PLF antibody and normal IgG were dialyzed with Slide-A-Lyzer Dialysis Cassette (3.5K MWCO) (Thermo Scientific Inc., Rockford, IL) to remove sodium azide before they were added to ASC-CMs. To confirm PLF effect in prostate cancer cells, we performed the same cell viability assay with ASC-CM after adding anti-PLF antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Tubule formation assay

The tubule formation assay was performed as described previously (Tahir, Yang et al. 2008). Briefly, HUVEC were trypsinized counted and plated on Matrigel™ Matrix (growth factor-reduced matrigel; BD Biosciences) coated 24 well-plates in EGM-2 (Lonza, Inc., Walkersville, MD) medium containing 2% FBS. After 16–24 h of incubation at 37°C in 5% CO_2, images of the tubules formed were captured by phase contrast microscopy. The tubule lengths of the endothelial network was measured by image analysis of five low-power fields using Nikon's NIS-Elements AR 3.0 imaging and quantification software.

Transwell invasion assay

Transwell invasion assay was performed using BioCoat™ Matrigel™ invasion chambers (BD Bioscience, San Jose, CA) according to manufacturer's instruction. Briefly, $5.0x10^3$ of RM-9 cells were seeded in the upper chambers (the inserts with 8.0 μm membrane) of 24-well plate with 500μL serum free growth medium, and 700 μL of each subgroup ASC-CM were added to the lower chambers. After RM-9 cells were allowed to invade to the bottom of the transwell for 24 hours, the invaded cells on the bottom surface of membrane were stained with HEMA 3 stain kit (Fisher Scientific, Kalamazoo, MI) according to manufacturer's protocol. The invading cells were counted under the microscope at 10x magnification.

Statistical analysis.

The statistical significance of the differences between the cell growth of Glipr1 WT and KO ASCs on different cell lines was determined by using two-tailed Student's *t* testing. The level of significance was set at $P < 0.05$. Data are expressed as means ± SE.

Results

Loss of Glipr1 enhanced Castration induced PLF expression/secretion in ASC/ASC-CM

To identify the factors secreted in response to castration and *Glipr1* gene status, we performed microarray assay using isolated RNA from each ASC subtype. PLF (Prolactin family2, subfamily C, member 2 and 3) were upregulated in the castrated ASC of both genotypes compared to the shamed genotypes. Beside cholecystokinin, PLF was consistently found to be upregulated in *Glipr1*$^{-/-}$ ASC when compared to *Glipr1*$^{+/+}$ ASC and in castrated *Glipr1*$^{-/-}$ ASC compared with shamed *Glipr1*$^{-/-}$ ASC (data not shown).

qRT-PCR analysis showed surgical castration reduced statistically significant amount of *Glipr1* mRNA (38%) in *Glipr1*$^{+/+}$ ASC (CxWT) compared to the shamed *Glipr1*$^{+/+}$ ASC(ShWT). Next, we detected that mRNA level of PLF was increased not only in ASC from *Glipr1*$^{-/-}$ (CxKO), but also after castration in both genotypes (CxKO> CxWT> ShKO> ShWT). Increased amount of PLF mRNA expression was significantly higher in castrated *Glipr1*$^{-/-}$ ASC subtype than other subtypes (Fig.1A). Western blotting analysis showed higher PLF protein expression in the ASC lysates from surgically castrated mice compared with surgically shamed mice, even though not much differences were detected between *Glipr1*$^{-/-}$ and *Glipr1*$^{+/+}$ ASC. However, ASC-CM from castrated *Glipr1*$^{-/-}$ (CxKO) ASC subtype expressed highest level of PLF secretion, and this data supports the qRT-PCR data above (Fig.1B).

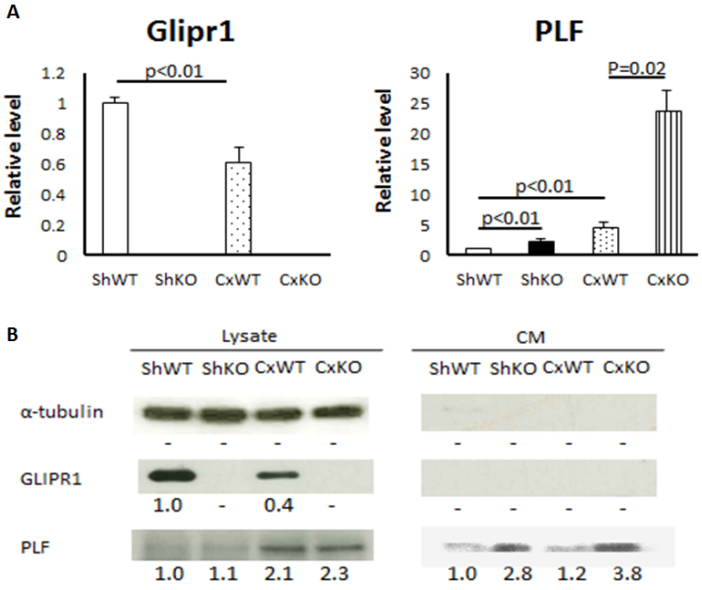

Fig. 1. **(A)** qRT-PCR with the primer specific for Glipr1 or PLF. Bar graph represents ratio relative to the amount of mRNA for each ASC to the shamed *Glipr1*$^{+/+}$ (ShWT). The amount of Glipr1 mRNA reduced approximately 40% after the castration. The amount of PLF mRNA in ASC subtypes increased in association with castration and *Glipr1* status. *Glipr1*$^{-/-}$ ASC (cxKO) showed the highest expression of PLF. **(B)** Expression of Glipr1 and PLF protein in ASC lysates and ASC-CM subtypes by western blotting. Expression of Glipr1 protein was reduced after castration in cxWT. PLF protein increased in association with castration and *Glipr1* status: cxKO showed the highest expression of PLF. α-tubulin was used as loading control.

PLF induced EC proliferation and tubule formation.

Since secreted PLF has been associated with angiogenesis in endothelial cells and many malignancies, ASC-CM from the four subtype were added separately to HUVEC cells and the effect of cell proliferation and tubule formations were compared among the four subtypes. Significantly increased proliferation and tubule formation of HUVEC

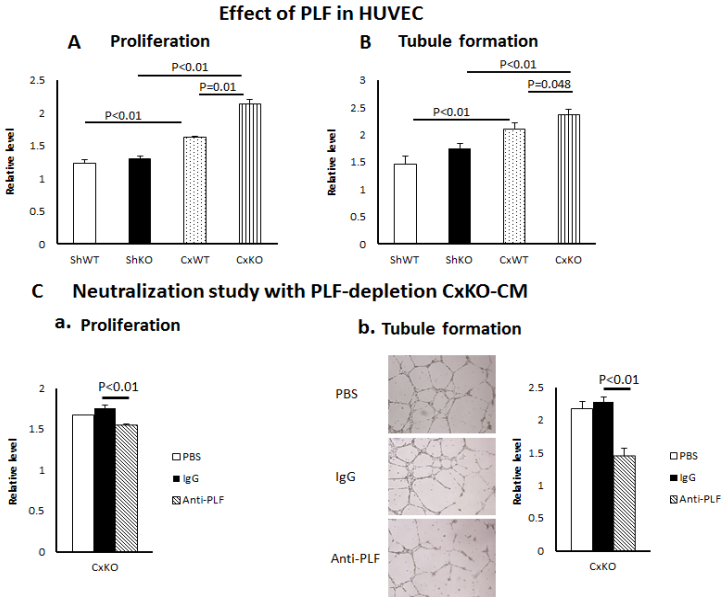

Fig. 2. **(A)** Effect of ASC-CM on HUVEC proliferation. CM collected from the cell sources indicated under columns was added to HUVEC. The number of living cells were measured using MTS assay. **(B)** Effect of ASC-CM on HUVEC tubule formation measured after 16–24 h. **(C)** ASC-CM pretreatment with PLF antibody decreased HUVEC proliferation (a) and tubule formation (b) stimulated by ASC-CM from castrated *Glipr1*$^{-/-}$ (CxKO) mice compared with ASC-CM treated with PBS or normal goat IgG. Images of the tubules formed were captured by phase contrast microscopy. The tubule lengths in each well were measured in 5 low-power fields. Bar graphs represent value or tubule length ratio relative to those in cells treated with serum free medium. Bars; SE.

were found in the cells that were grown with ASC-CM from castrated *Glipr1*$^{-/-}$ (CxKO) males compared to the ASC-CM from *Glipr1*$^{+/+}$ (CxWT) or shamed *Glipr1*$^{-/-}$ (SjKO) and *Glipr1*$^{+/+}$ (ShWT) males (Fig.2A,B). When we added ASC-CM from castrated *Glipr1*$^{-/-}$ males to HUVEC after it was neutralized with PLF antibody, we found reduced cell proliferation and the tubule formation. In fact, the reduction of the cell proliferation is statistically significant compared with the IgG control ($P< 0.01$) and the tubule formation was also significantly reduced compared with PBS and IgG control groups ($P< 0.01$ for both). This data confirms the cellular effect of PLF on proliferation and tubule formation of HUVEC (Fig.2Ca, 2Cb).

PLF promoted prostate cancer cell growth.

Next, to examine whether ASC-CM alters prostate tumor cell behavior, we performed the proliferation and the invasion assay of RM-9 (Fig.3A, 3B). As in the HUVE cells, we detected CM from castrated *Glipr1*$^{-/-}$ (CxKO) ASC promoted significantly increased cancer cell growth and invasion compared with ASC-CMs from *Glipr1*$^{+/+}$ (CxWT) or shamed *Glipr1*$^{-/-}$ (ShKO) and *Glipr1*$^{+/+}$ (ShWT) males. We also observed that pre- PLF antibody treatment of the ASC-CMs in the four subtypes offsets the effect of PLF in RM-9 as seen with HUVEC above (Fig.3C). Reductions of RM-9 cell proliferation grown with CMs after pre-treatment by PLF antibody were statistically significant in all the subtypes except CM from shamed *Glipr1*$^{+/+}$ (ShWT) males.

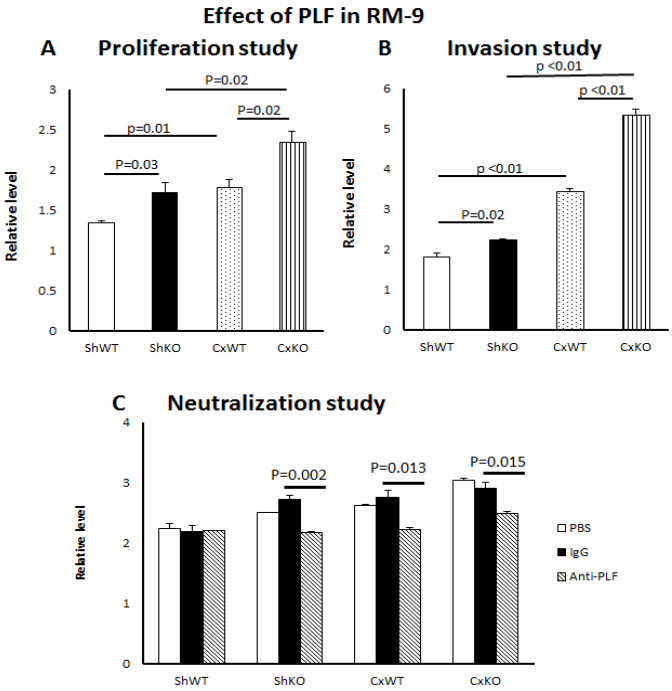

Fig. 3. **(A)** Effect of ASC-CM on mouse prostate cancer cell (RM-9) proliferation. CM collected from the cell sources indicated under columns was added to RM-9. The number of living cell measured using MTS assay. **(B)** Effect of ASC-CM on RM-9 invasion. RM-9 invasion was assayed as describe above. **(C)** Pre-treatment with PLF antibody decreased RM-9 proliferation. Each ASC-CM was either treated with PBS, normal goat IgG or anti-PLF antibody.

KEY RESEARCH ACCOMPLISHMENTS

1. In microarray assay performed, PLF was consistently found to be upregulated in *Glipr1*$^{-/-}$ ASC when compared to *Glipr1*$^{+/+}$ ASC and in castrated *Glipr1*$^{-/-}$ ASC compared with shamed *Glipr1*$^{-/-}$ ASC.
2. Western blotting analysis showed higher PLF protein expression in the ASC lysates from surgically castrated mice compared with surgically shamed mice. However, ASC-CM from castrated *Glipr1*$^{-/-}$ ASC subtype expressed highest level of PLF secretion.
3. Significantly increased proliferation and tubule formation of HUVEC were found in the cells that were grown with ASC-CM from castrated *Glipr1*$^{-/-}$ males. These effects on HUVEC were reduced when the ASC-CM was pre-neutralized with PLF antibody.
4. CM from castrated *Glipr1*$^{-/-}$ ASC promoted highest cancer cell growth and invasion and these effects on RM-9 were reduced when the ASC-CM was pre-neutralized with PLF antibody.

REPORTABLE OUTCOMES

None to report at this time. We currently have a paper in preparation.

CONCLUSION

During this year, we isolated adipocyte stromal cells (ASC) from white adipose tissue (WAT) of *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice. ASC was also collected from surgically castrated a *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice. Microarray analysis was performed on the conditioned medium (CM) collected from the ASC of 4 subtypes (castrated *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice and shamed *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice). We found an angiogenic growth promoting factor, proliferin, secreted in the castrated *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice in higher amount than shamed *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$ male mice, and this amount was higher in castrated *Glipr1*$^{-/-}$ than castrated *Glipr1*$^{+/+}$. ASC-CM from the 4 subtypes was analyzed and their effects were compared using mouse prostate cancer cell line (RM-9) as well as normal HUVEC. We found CM-ASC from castrated *Glipr1*$^{-/-}$ mice significantly increased proliferation and tubule formation of HUVEC and cell growth and invasion of RM-9 mouse prostate cancer cells. These cellular effects were reduced when the ASC-CM was pre-neutralized with PLF antibody. Our major findings are that castration induces changes in WAT, initiating secretion of factors that may promote angiogenic activities and invasion of tumor cells.

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